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MEMORANDUM

TO: Sherry Knowles

FROM: Odessa Roberts *OR*

DATE: September 27, 1996

RE: English Translation of Netherlands Patent No. 8901258
EMU133 E2690/084065

As requested, attached is the English translation of the above-identified patent document. A copy of this document is being sent to the above-identified file.

Patent Board of The Netherlands

12A Deposit for Inspection 11 8901258

19 NL

54 5-Halogeno-2',3'-dideoxycytidine derivatives in medications for the treatment of retrovirus infections.

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The documents attached to this sheet are a copy of the originally submitted description with claim(s) and any drawing(s).

B Br/GT/28-REGA

5-Halogeno-2',3'-dideoxycytidine derivatives in medications for the treatment of retrovirus infections.

The invention concerns new dideoxycytidine derivatives and their application in a therapeutic agent for the treatment of retrovirus infections such as AIDS and AIDS-related diseases.

AIDS, or acquired immune deficiency syndrome, is a pandemic immunosuppressive disease, which is the result of an exhaustion of helper T-lymphocyte cells in the human body. The cause has been identified as a retrovirus and is called "human immunodeficiency virus," or HIV. At this moment, two types (HIV-1 and HIV-2) of that virus have been described; both types can cause AIDS or AIDS-related diseases, although HIV-1 is more widely distributed than HIV-2.

Many efforts to find suitable anti-HIV agents have already been put to work, and it has been reported that many chemicals and compounds counter the replication of HIV (generally type 1) in vitro. For an overview, see E. de Clercq, *Anticancer res.*, 7, 1023-1038 (1987).

Among the proposed anti-HIV compounds 3'-azido-2',3'-dideoxythymidine (azidothymidine or AZT) is at the moment the only compound which has proven clinically usable in the treatment of AIDS patients, compare Fischl et al, *New England J. Med.* 317, 185-191 (1987).

In tests in vitro, in addition to 3'-azido-2',3'-dideoxythymidine, 3'-fluoro-2',3'-dideoxythymidine has proven to be a powerful inhibitor of HIV replication, compare Balzarini et al, *Biochemical Pharmacology*, 37, 2847-2856 (1988). Further, one finds powerful anti-HIV action with 5-chloro-3'-azido- and 5-chloro-3'-fluoro derivatives of 2',3'-dideoxyuridine, compare Balzarini et al, *Biochemical Pharm.*, 38, 869-894 (1989).

In the cytidine series, 2',3'-dideoxycytidine appears to be a powerful anti-HIV inhibitor, as well as the 2',3'-didehydro derivative thereof, while the 3'-azido and 3'-fluoro derivatives thereof are less powerfully active, compare Balzarini et al., *l.c.*, 1988.

In advanced investigation, it has now been found that 5-halogeno-3'-azido, 5-halogeno-3'-fluoro, and 5-halogeno-2',3'-didehydro derivatives of 2',3'-dideoxycytidine have a powerful and selective anti-HIV action, which is comparable to that of 2',3'-dideoxycytidine. This means that the said compounds can be used advantageously in pharmaceutical preparations against retrovirus infections including hepatitis B.

The 5-halogeno-3'-azido, 5-halogeno-3'-fluoro, and 5-halogeno-2',3'-dideohydro derivatives of 2',3'-dideoxycytidine are new chemicals which can be synthesized via every usual route for nucleoside analogs. Preferably, a corresponding 2',3'-dideoxyuridine derivative is first made, which is converted to a 2',3'-dideoxycytidine derivative by a known method. The halogen atom in the 5-position is preferably only introduced when the substituent in the 3'-position is already present.

It is noted that by "halogeno," chloro, bromo, iodo, and fluoro are understood.

Several examples follow for the synthesis of the chemicals according to the invention.

Synthesis example 1

5-chloro-3'-azido-2',3'-dideoxycytidine.

2.6 g (4.95 mmol) 5'-O-monomethoxytrityl-3'-azido-2',3'-dideoxyuridine was converted with 1.0 g (7.5 mmol) N-chlorosuccinimide in 100 ml of pyridine. By refining and chromatographic purification, 2.52 g (4.5 mmol, 91%) of a light brown foam was obtained, which together with anhydrous pyridine was

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vaporized and dissolved in 50 ml of dichloroethane-pyridine (5:1). In 20 minutes, 20 ml of 10% solution of trifluoromethane sulphonic acid anhydride in 1,2-dichloroethane was dripped into the cooled solution (0°C). After three hours at room temperature, according to TLC (CHCl₃-MeOH 95:5), the basic material was completely converted. The mixture was poured into 200 ml methanol saturated with ammonia. The solution was stirred one night at room temperature, after which according to TLC in addition to the basic material a new product was present in almost equal quantities. After concentration, the residue was dissolved in ethyl acetate and washed with water and with brine. The organic layer was dried, evaporated dry and purified, whereby 1.00 g (1.78 mmol, 40%) reclaimed 5-chloro-5'-O-monomethoxytrityl-3'-azido-2',3'-dideoxyuridine and 1.26 g (2.25 mmol, 50%) 5-chloro-5'-O-monomethoxytrityl-3'-azido-2',3'-dideoxycytidine was obtained as foam. UV (MeOH) λ_{\max} 288 nm. After 30 minutes, this foam was treated at 60°C with 100 ml 80% acetic acid. After adsorption on silica gel, the mixture was purified (CHCl₃ to CHCl₃-MeOH 94:6) whereby 380 mg (1.32 mmol) of a light brown foam was obtained which crystallized out of MeOH diethyl ether. Yield 221 mg (0.77 mmol, 34%), smp.: 173-175°C (dec).

[nine illegible lines of formula]

Synthesis example 2

5-chloro-3'-fluoro-2',3'-dideoxycytidine

760 mg (2.47 mmol) 5'-chloro-5'-O-acetyl-3'-fluoro-2',3'-dideoxyuridine

was dissolved in 24 ml of dichloroethane-pyridine (5:1) and cooled in an ice salt bath. In 10 minutes, 10 ml of 10% solution of trifluoromethane sulphonic acid anhydride was dripped into dichloroethane, after which the mixture was stirred for 3 hours at ambient temperature. According to TLC (CHCl₃-MeOH 95:5), the basic material was completely converted. The contents were poured into 100 ml methanol saturated with ammonia and stirred for 15 hours. Afterwards, according to TLC (CHCl₃-MeOH 9:1), two nucleosidic products were present, of which the fastest moving product migrated with the deacylated basic material. By flash chromatographic purification (CHCl₃-MeOH 97:3 to 9:1), 286 mg (1.08 mmol, 43%) of 5-chloro-3'-fluoro-2',3'-dideoxyuridine was reclaimed and 600 mg of impure brown foam was obtained. After intensive purification, 148 mg (0.46 mmol, 22%) of the title compound was isolated as a white foam which crystallized out of MeOH acetone. Smp.: 179-180°C.

[eleven illegible lines of formula]

Synthesis example 3

5-chloro-2',3'-didehydro-2',3'-dideoxycytidine.
1.46 g (5.46 mmol) of 5'-O-propionyl-2',3'-didehydro-2',3'-dideoxyureidine was converted in a yield of 81% to 5'-O-propionyl-2',3'-didehydro-2',3'-dideoxycytidine with the help of triazole and 0-chlorophenyl dichlorophosphate, according to the

method of Sung for conversion of uridine derivatives to cytidine ones. Compare W. L. Sung, J. Org. Chem. 47, 3623-3628 (1982).
UV (MeOH) λ_{\max} 271 and 237 nm.

Reaction with benzoic acid anhydride in anhydrous pyridine gave the N-benzoilized product in a 93% yield. UV (MeOH) λ_{\max} 261 and 304 nm.

The protected nucleoside analog was treated for 30 minutes in pyridine at 100°C with 1.5 equivalents of N-chloro-succinimide. Intensive refining produced 38% of the 5-chloro product in addition to 21% reclaimed material. UV (MeOH) λ_{\max} 261 and 331 nm.

Deprotection with methanol saturated with ammonia finally produced 62% of the title product which crystallized out of methanol diethylether. Smp.: 144-145°C.

[nine illegible lines of formula]

In tests which led to the invention, it was found that the compounds from synthesis examples 1, 2, and 3 are able to inhibit the cytopathogenicity of HIV-1 in MT-4 cells in a 50% effective dose (ED₅₀) of 9µM, 14µM and 15µM respectively. They are almost as active against the replication of HIV-2. Furthermore, the compounds of synthesis examples 1 and 2 are barely poisonous to the MT-4 cells, so that they have a high selectivity index, as good as or even better than that of 2',3'-dideoxycytidine.

In tests of the ability to counter the transformation caused by the Moloney murine sarcoma virus (MSV) of murine C3H/3T3 fibroblasts, it

appeared that none of the derivatives

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from the synthesis examples showed any antiviral activities in concentrations to 1000 μM . This as opposed to azidothymidine which is an efficient inhibitor of MSV ($\text{ED}_{50}=0.027\mu\text{M}$) and to 2',3'-dideoxycytidine which has an ED_{50} of $10\mu\text{M}$. Nonetheless it is assumed that the said derivatives have sufficient antiviral activity against human retroviruses which infect human cells.

On the basis of these data, the said compounds are usable in medications against AIDS and AIDS-related diseases and in general in medications against retrovirus infections including hepatitis B.

Therapeutic preparations which contain any of the invented compounds as an active ingredient for the treatment of retrovirus infections, such as AIDS or AIDS-related diseases in human practice, can take the form of powders, suspensions, solutions, sprays, emulsions, salves or creams and can be used for local intranasal, rectal, and vaginal administration, as well as for oral or parenteral (intravenous, intradermal, intramuscular, intrathecal, etc.) administration. Such preparations can be obtained by combining the active compounds (e.g. by mixing, dissolving, etc.) with pharmaceutically acceptable neutral excipients (such as hydrous or anhydrous solvents, stabilizers, emulsifiers, detergents, additives) and further, if desired, with pigments and aromatic substances. The concentration of the active ingredient in the therapeutic preparation can vary strongly between 0.1% and 100%, depending on the manner of administration. The dose of the active ingredient to be administered can vary between 0.1 mg and 100 mg per kilogram of body weight.

The anti-HIV properties of the 5-halogeno-2',3'-dideoxycytidine derivatives according to the invention are documented by the following examples, which should not be read in a limiting sense. In them, 2',3'-dideoxycytidine and 3'-azido-2',3'-dideoxythymidine are used for comparison.

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The viruses used in the examples were HIV-1 and HIV-2, respectively obtained from the growth liquid of H9 cells sustainedly infected with HIV-1 and from the growth liquid of CEM cells sustainedly infected with HIV-2. Further, use was made of Moloney murine sarcoma virus (MSV), prepared from tumors which were caused by infection in vivo with three-day-old NMRI mice (compare De Clercq et al, Proc. Soc. Exp. Biol. Med., 137, 590-594, 1971).

The cells used in the examples were MT-4 cells as described by I. Miyoshi et al, Gann Monogr. 28, 219-218[sic] (1982). These cells were grown in a culture medium consisting of RPMI-1640 medium, supplemented with 20 mM Hepes buffer, 10% (v/v) inactivated fetal calf serum and 2 mM glutamine. This RPMI-1640 medium is a standard medium which contains anorganic salts such as NaCl, NaHCO_3 , Na_2HPO_4 , etc., as well as glucose, various amino acids and various vitamins.

Five different chemicals were used as test compounds, i.e.:
 AzddClCyd: 5-chloro-3'-azido-2',3',-dideoxycytidine,
 FddClCyd: 5-chloro-3'-fluoro-2',3',-dideoxycytidine,
 D4ClC: 5-chloro-2',3'-didehydro-2',3',-dideoxycytidine,
 ddCyd: 2',3',-dideoxycytidine,
 AzddThd: 3'-azido-2',3',-dideoxythymidine.

Example 1

Inhibiting of the cytopathogenic action of HIV-1.

The test compounds were valued on their inhibiting effect on the cytopathogenic action of HIV-1 in MT-4 cells.

In a first series of tests, MT-4 cells (5×10^5 cells/ml) were suspended in a fresh growth medium consisting of RPMI-1640 medium with, in addition to the named additions, also 0.075% (w/v) NaHCO_3 , 2.5 $\mu\text{g/ml}$ Fungizone (Squibb N.V.). The suspension was infected with a cell suspension of 200 CCID₅₀ of HIV-1 per mm (1 CCID₅₀ is the infective dose for 50% of the cell cultivation). Immediately after the infection,

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100 μl portions of the cell suspension were combined in the cavities of a test plate with 100 μl portions of appropriate dilutions of the test compounds. In this way, each cavity of 200 μl contained 20 CCID₅₀ of HIV and 5×10^4 MT-4 cells. After five days of incubating in 37°C in a humid atmosphere with control of the CO₂ content, the viable cells were counted.

A second series of tests, parallel to the first, was performed with non-infected cell cultures, which were incubated in the presence of different concentrations of the test compounds. Here also, the number of viable cells was counted afterwards. From the values found, the 50% effective dose (ED₅₀) and the 50% cytotoxic dose (CD₅₀) were calculated, that is to say, the concentrations of test compounds necessary to reduce by 50% the number of viable cells in the virus-infected and non-infected cell cultures respectively.

The results are reflected in table 1.

| Compound | ED ₅₀ | CD ₅₀ |
|-----------|------------------|------------------|
| AzddClCyd | 9 | 923 |
| FddClCyd | 14 | 1000 |
| D4ClC | 15 | 170 |
| ddCyd | 0.27 | 39 |
| AzddThd | 0.002 | 5.2 |

From the table, it is apparent that the three compounds from the synthesis examples 1-3 have a value for ED₅₀ of 9 μM , 14 μM and 15 μM respectively. Two of the three compounds have, furthermore, an extremely low toxicity (high value of CD₅₀) so that their therapeutic index will be just as great as ddCyd or even better.

Tests with HIV-2 showed the same picture.

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Example 2

Effect of various additions on the anti-HIV action.

The tests from example 1 were repeated in the presence of various additions, after which the effect of these additions was determined.

In a first series of tests, MT-4 cells (10^6 cells per ml) were suspended in a fresh culture medium such as the one named in example 1. The suspension was infected with a cell suspension of 200 CCID₅₀ of HIV-1 per ml. Afterwards, 50 μ l portions of the infected cell suspension were brought together in the cavities of a test plate with 100 μ l portions of an appropriate dilution of a test compound and 50 μ l portions of a medium which contained certain additions. After five days of incubating in 37°C in a humid atmosphere with control of the CO₂ content, viable cells were counted.

A second series of tests, parallel to the first, was performed with non-infected cell cultures. Here also, the number of viable cells was counted afterwards. From the values found, the values of ED₅₀ and CD₅₀ 50 were calculated, in the same manner as in example 1.

The additions used were:

dCyd 2'-deoxycytidine
dThd 2'-deoxythymidine
THU tetrahydrouridine
dTHU 2'-deoxytetrahydrouridine

The results, together with those of example 1, are stated in table 2.

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Table 2

| Compound | Addition | ED ₅₀ (μ M) | CD ₅₀ (μ M) |
|-----------|--|--------------------------------|--------------------------------|
| AzddClCyd | None | 9 | 923 |
| | dCyd (1mM) | >500 | >500 |
| | dThd (250 μ M) + dCyd (1mM) | ≥500 | >500 |
| | THU (250 μ g/ml) + dTHU (250 μ g/ml) | ≥500 | >500 |
| | dCyd (1mM) + THU (250 μ g/ml) + | >500 | >500 |
| | +dTHU (250 μ g/ml) | | |
| FddClCyd | None | 14 | >1000 |
| | dCyd (1mM) | >500 | >500 |
| | dThd (250 μ M) + dCyd (1mM) | 249 | >500 |
| | THU (250 μ g/ml) + dTHU (250 μ g/ml) | 248 | >500 |
| | dCyd (1mM) + THU (250 μ g/ml) + | >500 | >500 |
| | dTHU (250 μ g/ml) | | |

| | | | |
|---------|--|-------|-------|
| D4ClC | None | 15 | 170 |
| | dCyd (1mM) | >100 | 223 |
| | dThd (250µM) + dCyd (1mM) | >100 | 211 |
| | THU (250µg/ml) + dTHU (250 µg/ml) | >100 | 220 |
| | dCyd (1mM) + THU (250µg/ml) + dTHU (250µg/ml) | >100 | 213 |
| ddCyd | None | 0.27 | 39 |
| | dCyd (1mM) | 56 | >500 |
| | dThd (250µM) + dCyd (1mM) | 14 | >500 |
| | THU (250µg/ml) + dTHU (250 µg/l) | 14 | >500 |
| | dCyd (1mM) + THU (250µg/ml) + dTHU (250µg/ml) | 424 | >500 |
| AzddThd | None | 0.002 | 5.2 |
| | dCyd (1mM) | 0.07 | >>100 |
| | dThd (250µM) + dCyd (1mM) | 0.7 | >>100 |
| | THU (250µg/ml) + dTHU (250 µg/ml) | 0.002 | 4.3 |
| | dCyd (1mM) + THU (250µg/ml) + dTHU (250µg/ml) | 0.002 | 40 |

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From table 2, it is apparent that the addition of 1000 µM of dCyd resulted in a clear decline of the anti-HIV activity of the compounds from synthesis examples 1-3. A similar decline also occurred with addition of THU + dTHU and of dCyd+THU+dTHU.

In this regard, the compounds of the synthesis examples were comparable to ddCyd, or 2',3'-dideoxycytidine. On the other hand, the results are not comparable to those of azidothymidine, where an addition of dCyd or of dCyd + dThd did bring about a decline in anti-HIV activity, but an addition of THU+dTHU or of THU+dTHU+dCyd had no effect.

It further appears that the cytostatic activity of the compounds from synthesis examples 1 and 2 was reduced considerably by the additions, just as with 2',3'-dideoxycytidine, while the compound of synthesis example 3 shows a deviating behavior here.

Example 3

Inhibition of Moloney murine sarcoma virus (MSV)

The test compounds were valued on their inhibiting effect on the transformation of C3H mouse-embryo fibroblasts by Moloney murine sarcoma virus (MSV).

C3H cells were introduced in a dose of 20000 cells per ml into the cavities of a 48-cavity test plate. After 24 hours, the cell cultures were infected with 80 focus-forming units of MSV and 120 minutes later, the culture medium was replaced by 1 ml of fresh medium that contained various concentrations of test compounds. After six days, the transformation of the cell cultures was observed microscopically.

From the tests, it appeared that none of the compounds from synthesis examples 1-3 showed any antiviral action in concentrations to 1000 μ M. On the other hand, the transformation was effectively inhibited by AzddThd ($ED_{50}=0.027\mu$ M) while ddCyd showed an ED_{50} of 10 μ M.

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C L A I M S

1. Compound chosen from the group of 5-halogeno-3'-azido-2',3'-dideoxycytidine, 5-halogeno-3'-fluoro-2',3'-dideoxycytidine, and 5-halogeno-2',3'-didehydro-2',3'-dideoxycytidine.
2. 5-chloro-3'-azido-2',3'-dideoxycytidine.
3. 5-chloro-3'-fluoro-2',3'-dideoxycytidine.
4. 5-chloro-2',3'-didehydro-2',3'-dideoxycytidine.
5. Pharmaceutical preparation for use in the treatment of retrovirus infections, which preparation includes a compound from the group 5-halogeno-3'-azido-2',3'-dideoxycytidine, 5-halogeno-3'-fluoro-2',3'-dideoxycytidine, and 5-halogeno-2',3'-didehydro-2',3'-dideoxycytidine as active ingredient.
6. Pharmaceutical preparation according to claim 5, which preparation contains the active ingredient in a concentration between approximately 0.1 and approximately 100 % by weight.
7. Pharmaceutical preparation according to claim 5, which preparation has the form of a powder, suspension, solution, spray, emulsion, salve or cream.
8. Pharmaceutical preparation for use in the treatment of AIDS or AIDS-related diseases which preparation contains a compound from the group of 5-halogeno-3'-azido-2',3'-dideoxycytidine, 5-halogeno-3'-fluoro-2',3'-dideoxycytidine, and 5-halogeno-2',3'-didehydro-2',3'-dideoxycytidine as active ingredient.
9. Pharmaceutical preparation according to claim 8, which preparation contains the active ingredient in a concentration between approximately 0.1 and approximately 100 % by weight.
10. Pharmaceutical preparation according to claim 8, which preparation has the form of a powder, suspension, solution, spray, emulsion, salve or cream.
11. Method for the treatment of retrovirus infections which consists of one's administering a compound from the group of 5-halogeno-3'-azido-2',3'-dideoxycytidine, 5-halogeno-3'-fluoro-2',3'-dideoxycytidine, and 5-halogeno-2',3'-didehydro-2',3'-dideoxycytidine to a patient suffering from a retrovirus infection.

12. Method for the treatment of AIDS or AIDS-related diseases which consists of one's administering a compound from the group of 5-halogeno-3'-azido-2',3'-dideoxycytidine, 5-halogeno-3'-fluoro-2',3'-dideoxycytidine, and 5-halogeno-2',3'-didehydro-2',3'-dideoxycytidine to a patient suffering from AIDS or an AIDS-related disease.

13. Application of a compound chosen from the group of 5-halogeno-3'-azido-2',3'-dideoxycytidine, 5-halogeno-3'-fluoro-2',3'-dideoxycytidine, and 5-halogeno-2',3'-didehydro-2',3'-dideoxycytidine for the preparation of a pharmaceutical preparation against retrovirus infections and hepatitis B.

14. Application of a compound chosen from the group of 5-halogeno-3'-azido-2',3'-dideoxycytidine, 5-halogeno-3'-fluoro-2',3'-dideoxycytidine, and 5-halogeno-2',3'-didehydro-2',3'-dideoxycytidine for the preparation of a pharmaceutical preparation against AIDS and AIDS-related diseases.
